

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

Intyg
Certificate

Härmed intygas att bifogade kopior överensstämmer med de
handlingar som ursprungligen ingivits till Patent- och
registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of
the documents as originally filed with the Patent- and
Registration Office in connection with the following
patent application.



(71) Sökande APREA AB, Stockholm SE
Applicant (s)

(21) Patentansökningsnummer 0300812-5 ✓
Patent application number

(86) Ingivningsdatum 2003-03-24 ✓
Date of filing

REC'D	14 APR 2004
WIPO	PCT

Stockholm, 2004-03-26

För Patent- och registreringsverket
For the Patent- and Registration Office

Marita Öun

Marita Öun

Avgift
Fee

PRIORITY
DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

BEST AVAILABLE COPY

46 8 4291070

Ink. t. Patent- och reg.verket

2003-03-24

Huvudfaktori Kesson

USE OF CHEMICAL COMPOUNDS FOR PREPARING PHARMACEUTICAL COMPOSITIONS, AND A METHOD OF TESTING SUBSTANCES FOR MEDICAL EFFECT**5 Field of the invention**

The present invention relates to the use of certain compounds, capable of transferring wild type p53 from an inactive and non-functional conformation into its active conformation, for the preparation of a pharmaceutical composition for use in treating medical conditions wherein wt p53 exists in an inactive state defined herein, and especially malignant melanoma. The invention also relates to a method of testing of compounds for the above-mentioned ability, wherein the level of inactive wt p53 is monitored. The method can be carried out both *in vitro* and *in vivo*.

15 Background art

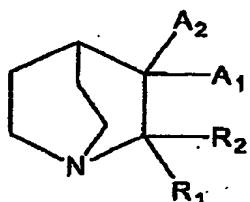
Although the tumour suppressor protein p53 is mutated in most human tumours, wild type (wt) p53 is dominant in malignant melanoma (Hollstein et al., *Science*, 253: 49-53, (1991); Sparrow et al., *Melanoma Res.* 1995, 5: 93-100 (1995); Hartmann et al., *Int J cancer*, 67: 313-317, (1996); and Petitclerc et al., *Cancer Res.*, 59: 2724-2730 (1999)), found Mab LM609, a function-blocking monoclonal antibody directed to $\alpha_v\beta_3$ integrin, to block M21 melanoma growth by inducing tumour apoptosis.

25 Malignant melanoma are typically refractory to apoptosis induction by chemical drugs or radioactivity.

Summary of invention

30 The present inventors have found certain compounds to be capable of inducing apoptosis in malignant melanoma cells, which compounds are defined in claim 1 as having a structure according to the formula I

46 8 4291070



2

Ink. t. Patent- och reg. verket

2003-03-24

Huvudfakten Klasser

(I)

wherein:

5 R_1 and R_2 are independently selected from hydrogen, hydroxymethyl, or a methylene group linked to the nitrogen atom of an amine-substituted phenyl group, to a nitrogen atom contained in the ring structure of a purine, 8-azapurine, or benzimidazol residue, or R_1 and R_2 may together represent a double bonded methylene group, and;

10 10 A_1 and A_2 are independently selected from hydrogen, hydroxyl, and benzyloxy, or A_1 and A_2 may together represent an oxygen atom being double bonded, with the proviso that when either of A_1 and A_2 is a benzyloxy group, both R_1 and R_2 are hydrogen.

15 15 Same compounds are also believed to be capable of inducing apoptosis in angiogenic vascular cells during angiogenesis, thereby inducing regression of angiogenic blood vessels and blocking various disease states dependent on blood vessel formation and/or vascular cell survival, including the growth and metastasis of various human tumours; adult blindness, e.g. caused by diabetic retinopathy or macular degeneration; psoriasis; states of chronic inflammation, e.g. rheumatoid arthritis; 20 20 and hemangioma.

25 25 The present inventors have also found a previously unknown and most unexpected inactive conformation state of wt p53 protein. The inactive wt p53 conformation has now been found in malignant melanoma cells. More importantly, said inactive wt p53 conformation can be reverted into a functional active conformation, capable of inducing apoptosis. Based on these findings, the present invention, in another aspect, provides a method of testing substances for their ability in transforming inactive wt p53 conformation into an active wt p53 conformation, and thus for finding suitable substances for treating malignant melanoma. The method is defined in 30 30 claim 7. Since same inactive wt p53 conformation also is believed to be present in angiogenic vascular cells during angiogenesis, same substances are also thought to

be useful in inhibiting angiogenesis in pathological conditions known to involve angiogenesis.

Brief description of the attached drawings

5

FIGURE 1A shows the percentage of apoptotic cells as function of time in human melanoma cells M21 expressing integrin $\alpha_v\beta_3$ and M21L lacking integrin $\alpha_v\beta_3$.

FIGURE 1B shows the percentage of apoptotic cells as function of time in human melanoma cells M0, lacking integrin $\alpha_v\beta_3$; M0-2baV, M0 cells overexpressing a

10 chimer of the extracellular domain of the integrin IIb subunit with the cytoplasmic tail of the integrin α_V subunit; and M0- α_V cells expressing high levels of the integrin α_V subunit.

FIGURE 1C illustrates the cell surface expression of integrin $\alpha_v\beta_3$ and the integrin β_3 subunit in different human melanoma cell lines.

15 FIGURE 1D shows the results of an analysis for cleavage of caspase-8 and caspase-9.

FIGURE 2A; upper panel depicts the specific p53 DNA binding activity in M21 and M21L cells collected from 3D-collagen using an electrophoretic gel shift assay (EMSA) with a p53 supershift using Pab 421; Lower panel presents the protein levels of p53 as compared to actin as a loading control.

FIGURE 2B, same as figure 2A upper panel, but with M0 and M0av cells.

FIGURE 2C shows acetylation of p53 at lysine 382.

Data shown in FIGURE 2D represents Pab 1620 and Pab 240 reactivity as percentage of D01 reactivity (total p53) in M21 ($\alpha_v\beta_3+$) cells and M21L ($\alpha_v\beta_3-$) cells.

25 FIGURE 3A shows the percentage of apoptotic cells in M21 cells ($\alpha_v\beta_3+$), M21L cells ($\alpha_v\beta_3-$) and a number of clones of M21L cells ($\alpha_v\beta_3-$) stably transfected with a dominant negative p53-His175, including M21Lp53His175-0, M21Lp53His175-1, M21Lp53His175-8 and M21Lp53His175-32.

FIGURE 3B illustrates tumour growth *in vivo* of cells used in Figure 3A.

30 FIGURE 3C the graph displays values of mean tumour volumes over time out of 5-7 animals for each tumour cell type shown in the Figure.

FIGURE 3D depicts a scatter graph showing the distribution of tumour wet weights after 25 d of tumour growth in mice of M21, M21L, M21Lp53His175-0 and M21Lp53His175-8.

2003-03-24

Huvudfaxen Kassan

4

FIGURE 4A shows the results of examination of p53 conformation with Pab1620 and Pab240 in melanoma cells incubated with or without 100 μ M of PRIMA-1 for 5d.

5 In FIGURE 4B the results of analysis of apoptosis in M21 and M21L cells in 3D-collagen with or without PRIMA-1 treatment for 5 d are shown.

FIGURE 4C shows mean tumour volumes over time of M21 human melanoma cells grown in C57/BL nude mice treated with or without PRIMA-1 (100 mg/kg) for 6 d. In FIGURE 4D, the distribution of tumour wet weights after 4 weeks of melanoma tumour formation with or without PRIMA-1 treatment as described in Fig. 4C are 10 displayed.

Detailed description of the invention

15 p53 is mutated in most human tumours, but the rare p53-mutation frequency in malignant melanoma is puzzling. However, malignant melanoma express high levels of integrin $\alpha_v\beta_3$, an integrin that promotes melanoma cell survival (Montgomery et al., *PNAS* 91: 8856-8860 (1994); Petitclerc et al; 1999; Hsu et al., *Am J Pathol* 153: 1435-1442 (1998)) and suppresses p53-activity in endothelial cells (Strömbäck et al., *J. Clin. Invest.* 98:426-433 (1996); Strömbäck et al., *J. Biol. Chem.* 277: 13371-20 13374 (2002)). The present inventors now suggest that integrin $\alpha_v\beta_3$ suppresses melanoma wt p53-activity via induction of a previously unknown inactive, unfolded wt p53-conformation. Said integrin $\alpha_v\beta_3$ -regulated p53-activity has been found to govern melanoma cell survival and tumour growth. Dominant negative p53 (His175) was found to restore cell survival and tumor growth of melanoma cells lacking integrin $\alpha_v\beta_3$, suggesting that the integrin-mediated inactivation of p53 controls melanoma cell survival. The compound 2,2-bis(hydroxymethyl)-1-azabicyclo[2.2.2]octan-3-one, also referred to as PRIMA-1, previously known from WO0224692 to have the ability to revert mutant p53-conformation to an active state, surprisingly restored the active conformation of wt p53 in melanoma cells, induced apoptosis and 25 blocked melanoma tumor growth in a wt p53-dependent manner. These results may explain the lack of need for p53-mutations in malignant melanoma, and points to a novel principle for melanoma therapy.

30 Strömbäck, S. et al. in *J. Clin. Invest.* 98:426-433 (1996), cited above, the contents of which is incorporated in its entirety herein by reference, reported that prolifera-

2003-03-24

Huvudfaxeri Kassan

5

tive endothelial cells become apoptotic in response to antagonists of integrin $\alpha_v\beta_3$, leading to the regression of angiogenic blood vessels, and thereby blocking the growth of various human tumours, and that the blocking of integrin $\alpha_v\beta_3$ lead to activation of p53.

5

Based on the findings reported in the above paper, and the findings disclosed in the present application, the inventors also believe the present compounds to be capable of inducing apoptosis in proliferative endothelial cells during angiogenesis, thereby inducing regression of angiogenic blood vessels and blocking various disease states 10 dependent on blood vessel formation and/or vascular cell survival, including the growth and metastasis of various human tumours; adult blindness, e.g. caused by diabetic retinopathy or macular degeneration; psoriasis; states of chronic inflammation, e.g. rheumatoid arthritis; and hemangioma. Consequently, the same inactive wt p53 conformation as disclosed herein is also believed to be present in such 15 proliferative endothelial cells during angiogenesis.

While the inactive conformation of wt p53 in melanoma cells is believed to be induced by the integrin $\alpha_v\beta_3$, the mechanism underlying the induction of any similar inactive wt p53 states in other tissues could be different. However, since the actual 20 mechanism of action of the present compounds is not fully understood, it is also conceivable that the same compounds could be useful for treating other pathological conditions wherein inactive wt p53 is present.

Substances useful in treating other pathological conditions wherein inactive wt p53 25 is present can be found by means of the generic method of the present invention.

As used herein, the term "inactive wt p53 conformation" is used interchangeably with the term "inactive, unfolded wt p53 conformation" to designate a conformation of wt p53 which is reactive with the monoclonal antibody Pab 240, but does not react with the monoclonal antibody Pab 1620. Furthermore, the inactive conformation also has the ability of being transformed into an active wt p53 conformation by 30 means of the above chemical compound designated PRIMA-1. Inactive wt p53 conformation is further characterized as being incapable of binding to DNA, and incapable of mediating apoptosis.

Pab 240 has previously only been known to be reactive with mutant p53 under non-denaturing conditions, while being equally reactive with mutant p53 and wt p53 under denaturing conditions. However, the present inventors have now found Pab 240 also to be reactive to the inactive state of wt p53, defined herein, under non-denaturing conditions.

Integrin $\alpha_v\beta_3$ plays a critical role for cutaneous melanoma progression displaying increased expression in the vertically growth phase melanoma lesions compared to more benign radial growth phase lesions (Albeda et al., *Cancer Res.* 50: 6757-6764 (1990)). In experimental models, expression of integrin $\alpha_v\beta_3$ promotes melanoma cell survival, tumour growth and conversion from radial to the vertical growth phase while blocking of integrin $\alpha_v\beta_3$ inhibits melanoma tumour growth by inducing apoptosis (Felding-Habermann, *J Clin Invest.* 89: 2018-22 (1992); Petitclerc et al., 1999; Hsu et al., 1998), implicating that integrin $\alpha_v\beta_3$ functionally regulates melanoma cell survival. Indeed, integrin $\alpha_v\beta_3$ has also been found to promote melanoma cell survival in a 3-dimensional environment *in vitro* (Montgomery et al., 1994). But, it is still unclear how integrin $\alpha_v\beta_3$ can control melanoma cell survival.

The tumour suppressor protein p53 functionally induces apoptotic cell death in response to various types of stresses (Oren, *Cancer Biol.* 5: 221-227 (1994); Ko and Prives, *Genes Dev.* 10: 1054-1072 (1996); Levine, *Cell* 88: 323-331 (1997)). Activated p53 can trigger apoptosis via two pathways (Vousden, *Cell* 103: 691-694 (2000)). One of them is associated with death receptors and activation of caspase-8 (Ashkenazi and Dixit, *Science* 281: 1305-8 (1998)), while the other apoptosis pathway is mediated through mitochondrial cytochrome c release and subsequent activation of Apaf1 and caspase-9 (Green and Reed, *Science* 281: 1309-12 (1998)). Furthermore, p53 acts as a specific DNA binding protein to activate or repress expression of a variety of genes, including transcriptionally activating bax (Miyashita et al., *Cell* 80: 293 (1995)), PIG3 (p53-inducible gene 3, Venot et al., *EMBO J.* 17: 4668-79 (1998)) and PUMA (p53 upregulated modulator of apoptosis, Nakano and Vousden, *Mol Cell.* 7: 683-694 (2001)) and transcriptionally repressing bcl-2 (Zhan et al., *Oncogene* 9: 3743-51 (1994)). These downstream targets are functionally involved in certain p53-mediated apoptotic processes.

5 A new mechanism for p53-induced apoptosis acting directly on the mitochondrial membrane has recently been reported by Mihara, A. et al. in "p53 has a direct apoptogenic role at the mitochondria", *Molecular Cell*, published, 27 January 2003. In accordance therewith, p53, by moving rapidly to the mitochondria, effectively 'jump starts' and amplifies its slower-starting transcription-dependent effect on apoptosis.

10 In addition, an antagonist of integrin $\alpha_v\beta_3$ induces proliferative endothelial cell apoptosis, which is associated with activation of p53 (Strömbäck et al., 1996; Strömbäck et al., 2002). Although p53 is mutated in most human tumors, wt p53 is dominant in malignant melanoma (Hollstein et al., 1991; Sparrow et al., 1995; Hartmann et al., 1996). In addition, malignant melanoma are typically refractory to apoptosis induction by chemical drugs or radioactivity.

Experimental

15

In order to demonstrate that integrin $\alpha_v\beta_3$ -mediated regulation of p53 regulates melanoma cell survival, the following experimentation, which will be described in more detail hereinafter, was performed. First, M21 human melanoma cells expressing integrin $\alpha_v\beta_3$, and the M21L subpopulation lacking integrin $\alpha_v\beta_3$, were analyzed in a 3-dimensional collagen model mimicking the pathophysiological dermal environment. It was found that integrin $\alpha_v\beta_3$ inhibited p53 activity by inducing an inactive wt p53 conformation. Furthermore, overexpression of a dominant negative p53 mutant (His175) restored M21L ($\alpha_v\beta_3$ -) cell survival and tumour growth, suggesting that p53 is a key target for integrin $\alpha_v\beta_3$ promoting melanoma cell survival. Importantly, PRIMA-1 could restore the active conformation of wt p53 in M21 cells and thereby induce melanoma cell apoptosis and suppress melanoma tumour growth. These results reveal a novel principle for treatment of melanoma by reactivation of wt p53.

30

Integrin $\alpha_v\beta_3$ promotes melanoma cell survival

With reference to Figure 1A, the maternal human melanoma cells M21, expressing integrin $\alpha_v\beta_3$, and a subpopulation, M21L, lacking integrin $\alpha_v\beta_3$, were cultured in a three-dimensional collagen gel (3D-collagen). At indicated times, apoptosis was detected by FITC-Annexin-V staining. FACS analysis showed that M21L cells lacking

$\alpha_v\beta_3$ became apoptotic to a much higher degree than M21 cells ($\alpha_v\beta_3+$), suggesting that integrin $\alpha_v\beta_3$ expression is critical for melanoma cell survival.

The maternal human melanoma cells M0, lacking integrin $\alpha_v\beta_3$; M0-2baV, M0 cells overexpressing a chimera of the extracellular domain of the integrin IIb subunit with the cytoplasmic tail of the integrin α_V subunit; and M0- α_V cells expressing high levels of the integrin α_V subunit, were cultured in a 3D-collagen. At indicated times, apoptosis was examined by FITC-Annexin-V staining. As shown in Figure 1B, FACS analysis showed that M0 α_V cells (expressing $\alpha_v\beta_3$) survived well while both M0 and M02baV cells underwent apoptosis to a higher degree at all time points. This indicates that the integrin α_V -subunit extracellular ligand binding part is important for melanoma cell survival.

Cell surface expression of integrin $\alpha_v\beta_3$ and the integrin β_3 subunit was analyzed in the human melanoma cell lines M21, M21L, M0, M0- α_V and M0-2baV stained with anti- $\alpha_v\beta_3$ Mab LM609 and anti- β_3 Mab AP3, respectively. As can be seen from Figure 1C, FACS analysis showed that both $\alpha_v\beta_3$ and the β_3 subunit were expressed in all M21 and M0 α_V cells but rarely expressed in M21L and M0 cells. While integrin β_3 was expressed in virtually all M0-2baV cells, integrin $\alpha_v\beta_3$ was not present.

It has been suggested that unligated integrin $\alpha_v\beta_3$ might cause apoptosis by direct induction of caspase-8 cleavage (Stupack et al, *J. Cell Biol.* 155: 459-470 (2001)). Therefore, lysates prepared from M21 and M21L cells out of 3D-collagen after 3, 5 and 7 days were analysed by Western blotting for cleavage of caspase-8 and caspase-9. With reference to Figure 1D, no cleavage of caspase-8 in these melanoma cells was observed, while a control with cycloheximide-treated Jurkate cells displayed caspase-8 cleavage. However, caspase-9 was cleaved to a higher degree in M21L cells ($\alpha_v\beta_3 -$) as compared to M21 ($\alpha_v\beta_3 +$) cells, indicating that lack of integrin $\alpha_v\beta_3$ caused apoptosis in M21L cells and that this apoptosis might be mediated by a mitochondrial apoptotic pathway involving caspase-9 but did not involve any detectable activation of caspase-8.

Integrin $\alpha_v\beta_3$ induces an unfolded conformation of wt p53 and suppresses wt p53 activity

The specific p53 DNA binding activity was examined in M21 and M21L cells collected from 3D-collagen using an electrophoretic gel shift assay (EMSA) with a p53 supershift using Pab 421. The p53 activity was similar in M21 and M21L cells at day 0. However, as shown in upper panel of Figure 2A, M21L cells lacking integrin $\alpha_v\beta_3$ displayed an increased p53 activity after culturing in 3D-collagen while M21 cells ($\alpha_v\beta_3+$) did not.

The protein levels of p53 were analyzed by Western blotting using anti-p53 Pab. With reference to lower panel of Figure 2A, the p53 protein levels in M21 and M21L cells showed no difference within 7 d in 3D-collagen (examination performed on day 0, 3, 5 and 7). The levels of actin were examined as a loading control. This indicates that integrin $\alpha_v\beta_3$ regulates p53 activity without influencing p53 protein levels.

The specific p53 DNA binding activity was examined in M0 and M0aV cells after incubation within 3D-collagen for 5 and 7 days, respectively. The EMSA assay showed that the p53 activity was higher in M0 cells lacking integrin $\alpha_v\beta_3$ than that in M0aV cells ($\alpha_v\beta_3+$) (see Figure 2B). This verifies the role for integrin $\alpha_v\beta_3$ regulating melanoma cell p53 activation state in a second cell system.

Acetylation of p53 at lysine 382, a known acetylation site for p53 activation was examined by Western blotting using anti-acetylation p53-L382 pab. As can be seen from Fig. 2C, the levels of acetylated p53 were reduced in M21 cells ($\alpha_v\beta_3+$) as compared to M21L cells ($\alpha_v\beta_3-$), while total p53 levels remained the same.

With reference to Figure 2D, the conformation of p53 was analyzed by ELISA using Pab1620 recognizing the active, folded p53 conformation; Pab 240 recognizing an inactive, unfolded p53 conformation and; DO1 recognizing both folded and unfolded p53 conformations. Data shown in Figure 2D represents Pab 1620 and Pab 240 reactivity as percentage of D01 reactivity (total p53). It was found that both M21 cells ($\alpha_v\beta_3+$) and M21L ($\alpha_v\beta_3-$) displayed an active p53 conformation in regular culture. However, p53 switched to an inactive unfolded p53 conformation after incubation in 3D-collagen-incubated M21 cells ($\alpha_v\beta_3+$), while M21L ($\alpha_v\beta_3-$) maintained an active p53 conformation. This suggests that integrin $\alpha_v\beta_3$ regulates wt p53 conformation in melanoma cells in a pathophysiological 3-dimensional environment.

46 8 4291070

Ink t Patent- och reg.verket

2003-03-24

10

Huvudfaxon Kassan

and that the lack of p53-activity in cells expressing integrin $\alpha_v\beta_3$ is caused by an unfolded, inactive p53-conformation.

5 **Integrin $\alpha_v\beta_3$ -regulated p53 controls melanoma cell survival and melanoma tumor growth**

M21L cells ($\alpha_v\beta_3^-$) were stably transfected with a dominant negative p53-His175 and a number of clones, including M21Lp53His175-0, M21Lp53His175-1, M21Lp53His175-8 and M21Lp53His175-32 were identified by examining p53 activity using EMSA after incubation in 3D-collagen for 5 d. The p53 activity in these clones was reduced to a similar level as in M21 cells expressing $\alpha_v\beta_3$ (boxed in Figure 3A). The M21L-p53His175 clones, M21L and M21 cells were incubated within 10 3D-collagen and apoptosis was examined by FITC-Annexin-V staining at times indicated in Figure 3A. FACS analysis showed that the number of apoptotic cells was 15 lower in the M21L carrying dn p53 clones as compared to M21L, and that the rate of apoptosis in the M21L-dnp53 clones was similar to that in M21 cells expressing integrin $\alpha_v\beta_3$, indicating that the over-expression of dominant negative p53 could rescue M21L cells from apoptosis to a similar degree as expression of integrin $\alpha_v\beta_3$ itself.

20

Role of integrin-regulated p53 in melanoma tumour growth was investigated *in vivo*. Results are shown in Figure 3B. Melanoma cells (1×10^6), including M21L, M21 and M21L-dn p53 clones M21Lp53His175-0 and M21Lp53His175-8, were injected s.c. into the back of 6-week-old C57/BL nude mice. Each type of injected cell 25 type included 7 mice. The tumours were allowed to grow for 4 weeks. Tumours were found to be formed by M21Lp53His175-0, M21Lp53His175-8, and M21 cells ($\alpha_v\beta_3^+$), while M21L cells lacking integrin $\alpha_v\beta_3$ typically did not form tumours within this time.

30 Tumour volumes were monitored for melanoma growth, as described in the preceding paragraph. Tumour volume over time was determined by the formula: width² × length × 0,52. With reference to Figure 3C, the displayed values are mean tumour volumes out of 5-7 animals for each tumour cell type. In the Figure, ◆ represents M21; □ M21L; ▲ Lp53His175-0; and ★ Lp53His175-8. M21Lp53His175-0 and 35 M21Lp53His175-8 formed large tumours similar in size to M21 tumours. However,

M21L cells lacking integrin $\alpha_v\beta_3$ typically grew only minimal or developed no tumours.

Scatter graph showing the distribution of tumour wet weights of M21, M21L,
5 M21Lp53His175-0 and M21Lp53His175-8 are contained in Figure 3D. Cells were
grown s.c. in nude mice for 4 weeks as described in Fig 3B.

Example

10 In this example it will be demonstrated that PRIMA-1 induces an active p53 conformation in integrin $\alpha_v\beta_3$ positive melanoma cells, promotes melanoma cell apoptosis and suppresses melanoma tumour growth *in vivo*.

PRIMA-1 is a substance known from WO0224692 to be able to reactivate the apoptosis-inducing function of mutant p53 proteins. To test if PRIMA-1 could affect also the unfolded, inactive conformation of wt p53 in melanoma cells enforced by integrin $\alpha_v\beta_3$, M21 cells ($\alpha_v\beta_3^+$) and M21L cells ($\alpha_v\beta_3^-$) were incubated with or without 100 μ M PRIMA-1 in 3D-collagen for 7 d. The p53 conformation was examined as described above. The results are presented in Figure 4A. As can be seen, PRIMA-1 could restore active p53 conformation from an unfolded inactive state in M21 cells ($\alpha_v\beta_3^+$), while the p53 conformation in control M21L cells ($\alpha_v\beta_3^-$) was not influenced by PRIMA-1. This demonstrates in an independent way that wt p53 in melanoma cells can undergo conformational changes between unfolded and folded states. More importantly, these results surprisingly indicates that wt p53 unfolded conformation in melanoma cells can be reverted to active conformation by PRIMA-1.

30 Apoptosis was analyzed in M21 cells in 3D-collagen with or without PRIMA-1 treatment for 5 d. Results are shown in Figure 4B. PRIMA-1 promoted apoptosis in M21 cells ($\alpha_v\beta_3^+$), suggesting that regulation of the p53 activation state controls melanoma cell survival, verifying the results using dn p53 (Fig 3) in an independent manner.

To test if restoration of an active wt p53 conformation by PRIMA-1 could also induce melanoma cell apoptosis *in vivo* and thereby block melanoma tumour growth, 35 M21 cells ($1,5 \times 10^6$) were injected s.c. into the back of 6-week-old C57/BL nude

mice. The mice were treated with or without PRIMA-1 (100 mg/kg) for 6 d starting 6 d after tumour inoculation. The graph in Figure 4C shows mean tumour volumes calculated as described above for four mice in each group. PRIMA-1 significantly inhibited M21 melanoma growth as compared to PBS control.

5

In Figure 4D, the distribution of tumour wet weights after 4 weeks of melanoma tumour formation with or without PRIMA-1 treatment as described in Fig. 4C are displayed. The finding that PRIMA-1 can suppress tumour growth of melanoma cells carrying wt p53 points to a novel principle for treatment of malignant melanoma by re-activating an inactive conformation of wt p53, which was kept inactive by integrin $\alpha_v\beta_3$.

10

Based on these unexpected findings the present inventors also expect structural analogues of PRIMA-1, such as PRIMA-2 and PRIMA-3, which have been described in WO0224692, as well as further analogues of PRIMA-1 described in International Patent Application No. PCT/SE03/00206 (not published) to exhibit similar activity as PRIMA-1.

20

In the compounds of formula I above, the phenyl group or the nitrogen-containing ring structure of R1, and the benzyloxy group of either of A₁ and A₂ can optionally be substituted, such as for example with halogen, methyl, methoxy, amino and/or halomethyl containing 1-3 halogen atoms.

25

The term halogen or halo refers to a fluorine, chlorine, bromine or iodine atom, of which chlorine generally is preferred. A compound of the invention may be in free form, e.g., amphoteric form, or in salt, e.g., acid addition or anionic salt, form. A compound in free form may be converted into a salt form in an art-known manner and vice-versa.

30

The pharmaceutically acceptable salts of the compounds of formula I (in the form of water, or oil-soluble or dispersible products) include the conventional non-toxic salts or the quaternary ammonium salts of these compounds, which are formed, e.g., from inorganic or organic acids or bases. Examples of such acid addition salts include acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, diglu-

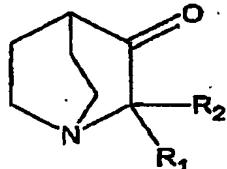
35

conate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, paemoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate. Base salts include ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine, lysine, and so forth. Also, the basic nitrogen-containing groups may be quaternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl; and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides like benzyl and phenethyl bromides and others.

Preferred specific examples of the compounds which can be used according to the present invention are 2,2-bis(hydroxymethyl)-1-azabicyclo[2.2.2]octan-3-one (also referred to as PRIMA-1), 9-(azabicyclo[2.2.2]octan-3-one)-6-chloro-9H-purine (also referred to as PRIMA-2), 2-(hydroxymethyl)quinuclidine-3,3-diol (also referred to as PRIMA-3), 2-(adenine-9-methylene)-3-quinuclidinone, 2-methylene-3-quinuclidinone, 2-(2-amino-3-chloro-5-trifluoromethyl-1-methylaniline)-3-quinuclidinone, 2-(6-trifluoromethyl-4-chlorobenzimidazole-1-methylene)-3-quinuclidinone, 2-(6-methoxypurine-9-methylene)-3-quinuclidinone, 2-(8-azaadenine-9-methylene)-3-quinuclidinone, 1-azabicyclo[2.2.2]oct-3-yl benzoate, 2-(5,6-dimethyl-benzimidazole-1-methylene)-3-quinuclidinone, 2-(8-azaadenine-7-methylene)-3-quinuclidinone, 2-(7-methylene-1,3-dimethyluric acid)-3-quinuclidinone, or 2-(2,6-dichloro-9-methylenepurine)-3-quinuclidinone, or a pharmaceutically acceptable salt thereof.

30

More preferably, the substance of the present invention has the structure of the following general formula I'



(I')

wherein

5 one of R₁ and R₂ is hydrogen and the other is a methylene group linked to the nitrogen atom of an amine-substituted phenyl group, to a nitrogen atom contained in the ring structure of a purine, 8-azapurine, or benzimidazol residue, and, more preferably the methylene group is linked to a nitrogen atom contained in the ring structure of a purine, 8-azapurine, or benzimidazol residue.

10 More preferably, one of R₁ and R₂ in formula I and I' is hydrogen, or both R₁ and R₂ are hydroxymethyl groups.

The following compounds are believed to exhibit an activity similar or greater than that of PRIMA-1: 2-(5,6-dimethyl-benzimidazole-1-methylene)-3-quinuclidinone, 2-(8-azaadenine-7-methylene)-3-quinuclidinone, 2-(7-methylene-1,3-dimethyluric acid)-3-quinuclidinone, 2-(2,6-dichloro-9-methylenepurine)-3-quinuclidinone and 2-(6-methoxypurine-9-methylene)-3-quinuclidinone.

20 General procedure for testing substances for ability of transferring wild type p53 from its inactive conformation into an active conformation

The inventive methods of testing substances are based on detection of wild type p53 in inactive conformation, either in a direct or indirect manner, as will be explained below.

25 In the methods of the present invention it is essential that only wild type and not mutant p53 is present. Accordingly, in any cells used in the method, only wild type p53 should be expressed, i.e. the p53 gene must not be mutated. Expression of wt p53 can readily be determined by the person skilled in the art using any suitable conventional methods, such as sequencing, mass spectroscopy, DNA-binding tests etc.

Naturally, it is also essential that the inactive form of wt p53 is present during testing. The presence of the inactive form of wt p53 can then suitably be established in a direct manner by using Pab240.

5 More particularly, the present inventors have found that wt p53 is inactivated under the following conditions: In melanoma cells cultured *in vitro* in a 3-dimensional environment, such as 3D collagen; in melanoma cells in tissue culture after irradiation with UV or gamma radiation, and; in melanoma cells grown *in vivo* in mice. The inventors also predict that wt p53 will take an inactive conformation in angiogenic 10 vascular cells during angiogenesis and that the wt p53 inactive conformation is also present in additional cell types and tissues and in other cases can be induced by other means of treatment.

15 The method of monitoring any changes in the level of inactive wt p53 during testing is not critical according to the invention, as long as the method allows for detection of any substantial reduction in the level inactive wt p53 and a consequent induction of active p53 levels.

20 Based upon the above definition of the new inactive wt p53 state, and the present detailed description, the skilled person will be able to find suitable methods for detecting and/or monitoring the level of inactive wt p53 using common general knowledge. Such methods will generally be based on measurement of wt p53 conformation and/or activity state, as will be explained below in further detail.

25 During testing, the level of inactive wt p53 can for example be established by using a suitable conformational-specific antibody, recognising only the inactive form, such as the above-mentioned Pab240. Since the total cellular level of wt p53 is formed by the combined levels of inactive p53 and active p53, respectively, measurement of the active p53 conformation can also be used as an indirect measurement of inactive p53, i.e. an absence of active p53 indicates that p53 is instead inactive. Measurement of the active p53 conformation can also be performed using a 30 suitable conformational-specific antibody, such as Pab 1620. If desired, the total level of wt p53 can be measured by means of a suitable antibody, which does not discriminate between the inactive and active forms of wt p53, such as DO1.

Of course, other direct or indirect parameters reflecting the activity state of wt p53 could also be used. Accordingly, the activity state of wt p53 can for example be detected by measurement of p53-bindning to DNA, by absence of expression of reporter constructs, for example using GFP or luciferase as reporters, by measurement of 5 p53 target genes, e.g. PUMA-1, PIG-3, bax, bcl-2 and/or caspase-3, by using microarray-related techniques to analyse specific p53 target genes or gene expression patterns indicating p53 activity and/or by measuring the capability of wt p53 to induce apoptosis and/or growth arrest.

10 Also, as indicated in Fig 2C, specific p53 acetylation could also be used as an indication of p53 activity. Likewise, phosphorylation of p53 is also conceivable as an indication of p53 activity.

15 The use of antibodies is however presently preferred for being a practical and more direct method of detecting and monitoring inactive wt p53, especially when Pab240 is used.

20 The general method of testing substances for the ability of transferring wt p53 from an inactive conformation into an active conformation according to the present invention will thus include the following general steps:

- A. Providing cells carrying wt p53 wherein inactive wt p53 conformation is present;
- B. Exposing the cells to a substance to be tested; and
- C. Measuring the cellular level of inactive wt p53 conformation, for example by 25 any of the above-mentioned methods.

30 The above general method can be adapted to both *in-vitro* or *in-vivo* testing by exposing the cells in step B either *in vivo* or *in vitro* to the substance to be tested, as will be readily understood hereinafter.

Step C is typically performed both before and after exposing the cell in step B to a substance to be tested.

35 *In vitro* testing of substances for ability of transferring wild type p53 from its inactive conformation into an active conformation

In the case of *in-vitro* testing for substances capable of reverting an inactive p53 conformation to an active conformation, cells carrying wt p53 can be grown in a 3-dimensional environment and the conformation and/or activity state of p53 measured, for example by any of the methods mentioned above, with and without, respectively, the addition of a substance for testing. A substance is considered functional if it is found to be capable of changing the conformation and/or activity of inactive wt p53. Alternatively, cells with wt p53 could be irradiated in order to induce the inactive wt p53 conformation, exposed to a substance to be tested, and thereafter analysed for p53 conformation and/or activity. Alternatively, a cell naturally displaying an inactive wt p53 conformation could be utilized or an inactive wt p53 conformation could be induced by other means, the cell thereafter being exposed to a substance to be tested, and analysed for inactive p53 state.

15 A tissue, an organ, or a part thereof, from a human or from an animal could also be cultured, treated and used for the inventive testing as described for cells *in vitro* above. Although such models sometimes are referred to as *ex-vivo* models, when used in the inventive method of testing of substances herein, such method is considered to be a method of *in-vitro* testing.

20 Alternatively, *in-vitro* testing of substances could be performed in models of angiogenesis. In vitro examples include, but are not limited to, tube forming assays of endothelial cells, outgrowth of blood vessels from stem cells, outgrowth of blood vessels from an isolated vessel originating from an animal or human, or analysis of 25 endothelial cells in tissue culture.

An *in-vitro* testing procedure could thus for example include the following general steps:

- A. Providing cells carrying wt p53 *in vitro*, and inducing inactive wt p53 conformation if not already present;
- 30 B. Exposing the cells to a substance to be tested; and
- C. Measuring the cellular level of inactive wt p53 conformation, directly or indirectly for example by any of the above-mentioned methods.

Step C is typically performed both before and after exposing the cell in step B to a substance to be tested.

5 ***In vivo testing of substances for ability of transferring wild type p53 from its inactive conformation into an active conformation***

Likewise, wt p53 conformation and/or activity could be assessed *in vivo*. For example, melanoma cells xenografts could be used *in vivo* to test substances for their capacity of reverting an inactive p53 conformation to active p53. In this case, an animal carrying such tumour xenograft would be treated with a substance to be tested, and the effect of this substance on wt p53 conformation and activity measured as described above. Alternatively, testing of substances could be carried out in animal melanoma models, where melanoma is occurring naturally or is provoked within the animal by treatment e.g. by UV-radiation and/or by carcinogenic chemicals and/or by genetic modifications. Administration of test substance to animal can be performed i.v., i.p., s.c., intratumoural, or otherwise.

20 Alternatively, *in-vivo* testing of substances could be performed in models of angiogenesis. Examples of *in-vivo* models include, but are not limited to, stimulation of angiogenesis in the chick embryo chorioallantoic membrane, stimulation of angiogenesis by a transplant of extracellular matrix or comparable substances onto mice or onto another animal, stimulation of angiogenesis in the cornea by an angiogenic compound, stimulation of angiogenesis in the retina by hypoxia, analysis of developmental angiogenesis in the retina or elsewhere, and induction of angiogenesis by 25 a tumour xenograft or another type of xenograft. Measurement of angiogenesis is performed by a quantification of the number of blood vessels within the tissue and/or by quantification of vascular cell markers and/or by measurement of vascular cell apoptosis and/or by similar means.

30 An *in-vivo* testing procedure could for example comprise the following general steps:
A. Providing an animal expressing wt p53 in a tissue thereof, wherein inactive wt p53 conformation is present;
B. Exposing cells of the tissue of the animal *in vivo* to a substance to be tested; and

46 8 4291070

lnk. t. Patent- och reg.verket

2003-03-24

19

Huvudfaxen Kässan

C. Measuring the cellular level of inactive wt p53 conformation, directly or indirectly, for example by any of the above-mentioned methods.

Step C is typically performed both before and after exposing the cell in step B to a substance to be tested.

The animals are typically sacrificed after the above-described *in-vivo* testing of substances.

10
09
08
07
06
05
04
03
02
01

46 8 4291070

20

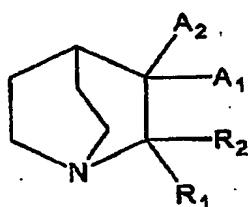
Ink t Patent- och reg.verket

2003 -03- 2 4

Huvudfaxes Kassan

Claims

1. Use of a compound capable of transferring wild type p53 from an inactive conformation thereof, which conformation is reactive to Pab 240 and not to Pab 1620, 5 into an active conformation capable of inducing apoptosis, which compound is selected from compounds having a structure according to the formula I



(I)

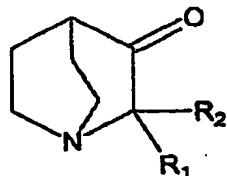
10 wherein:

R₁ and R₂ are independently selected from hydrogen, hydroxymethyl, or a methylene group linked to the nitrogen atom of an amine-substituted phenyl group, to a nitrogen atom contained in the ring structure of a purine, 8-azapurine, or benzimidazol residue, or R₁ and R₂ may together represent a double bonded methylene group, and;

15 A₁ and A₂ are independently selected from hydrogen, hydroxyl, and benzyloxy, or A₁ and A₂ may together represent an oxygen atom being double bonded, with the proviso that when either of A₁ and A₂ is a benzyloxy group, both R₁ and R₂ are hydrogen, 20 or a pharmaceutically acceptable salt thereof for the preparation of a pharmaceutical composition for use in treating medical conditions wherein wt p53 is inactive.

2. The use of claim 1, wherein the compound is selected from compounds having the structure of the general formula I'

25



(I')

wherein

46 8 4291070

Ink t. Patent- och registreringsverket

2003-03-24

21

Huvudfaxon Kessan

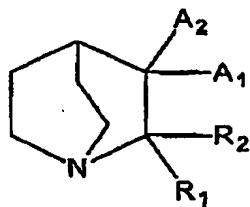
one of R_1 and R_2 is hydrogen and the other is a methylene group linked to the nitrogen atom of an amine-substituted phenyl group, to a nitrogen atom contained in the ring structure of a purine, 8-azapurine, or benzimidazol residue, and, more preferably the methylene group is linked to a nitrogen atom contained in the 5 ring structure of a purine, 8-azapurine, or benzimidazol residue.

3. The use of claim 1, wherein the compound is selected from 2,2-bis(hydroxymethyl)-1-azabicyclo[2.2.2]octan-3-one, 9-(azabicyclo[2.2.2]octan-3-one)-6-chloro-9H-purine, 2-(hydroxymethyl)quinuclidine-3,3-diol, 2-(adenine-9-methylene)-3-quinuclidinone, 2-methylene-3-quinuclidinone, 2-(2-amino-3-chloro-5-trifluoromethyl-1-methylaniline)-3-quinuclidinone, 2-(6-trifluoromethyl-4-chlorobenzimidazole-1-methylene)-3-quinuclidinone, 2-(6-methoxypurine-9-methylene)-3-quinuclidinone, 2-(8-azaadenine-9-methylene)-3-quinuclidinone, 1-azabicyclo [2.2.2]oct-3-yl benzoate, 2-(5,6-dimethyl-benzimidazole-1-methylene)-3-quinuclidinone, 2-(8-azaadenine-7-methylene)-3-quinuclidinone, 2-(7-methylene-1,3-dimethyluric acid)-3-quinuclidinone, or 2-(2,6-dichloro-9-methylenepurine)-3-quinuclidinone, or a pharmaceutically acceptable salt thereof.

4. The use of anyone of the claims 1-3 together with a pharmaceutically acceptable carrier, diluent and/or excipient.

5. The use of any of the previous claims, wherein the medical condition is malignant melanoma and/or a pathological condition involving undesired angiogenesis.

25 6. A method of treating malignant melanoma and/or inhibiting undesired angiogenesis, comprising administrating to a mammal in need thereof a pharmaceutically efficient amount of a compound selected from compounds having a structure according to the formula I



(I)

wherein:

R₁ and R₂ are independently selected from hydrogen, hydroxymethyl, or a methylene group linked to the nitrogen atom of an amine-substituted phenyl group, to a nitrogen atom contained in the ring structure of a purine, 8-azapurine, or benzimidazol residue, or R₁ and R₂ may together represent a double bonded methylene group, and;

A₁ and A₂ are independently selected from hydrogen, hydroxyl, and benzoxyloxy, or A₁ and A₂ may together represent an oxygen atom being double bonded, with the proviso that when either of A₁ and A₂ is a benzoxyloxy group, both R₁ and R₂ are hydrogen.

7. Method of testing compounds for the ability of transferring wild type p53 from an inactive conformation into an active conformation comprising the steps:

A. Providing cells carrying wt p53, in which cells inactive wt p53 conformation is present;

B. Exposing the cells to a substance to be tested; and

C. Measuring the cellular inactive wt p53 conformation.

8. The method of claim 7, wherein integrin $\alpha_v\beta_3$ is present in the cells.

20

9. The method of claim 7 or 8, wherein the Pab 240 is used for detecting wt p53 in its inactive conformation.

10. The method of any of the claims 7-9, wherein a compound of claim 1 is tested.

25

11. The method of any of the claims 7-10, wherein the cells in step B are exposed in vivo to the substance to be tested.

46 8 4291070

Ink. t. Patent- och reg.verket

2003-03-24

Huvudfaxes Klassen

ABSTRACT

The present invention relates to the use of certain compounds, capable of transferring wild type p53 from an inactive and non-functional conformation into an active conformation, for the preparation of a pharmaceutical composition for use in treating medical conditions wherein wt p53 exists in an inactive state defined herein, and especially malignant melanoma. The invention also relates to methods of *in vivo* and *in vitro* testing of compounds for the above-mentioned ability, wherein inactive wt p53 is monitored or detected.

10
9
8
7
6
5
4
3
2
1

46 8 4291070

1/11

Ink. t Patent- och reg.vi

2003-03-24

Huvudfaxen Kassar

Figure 1A

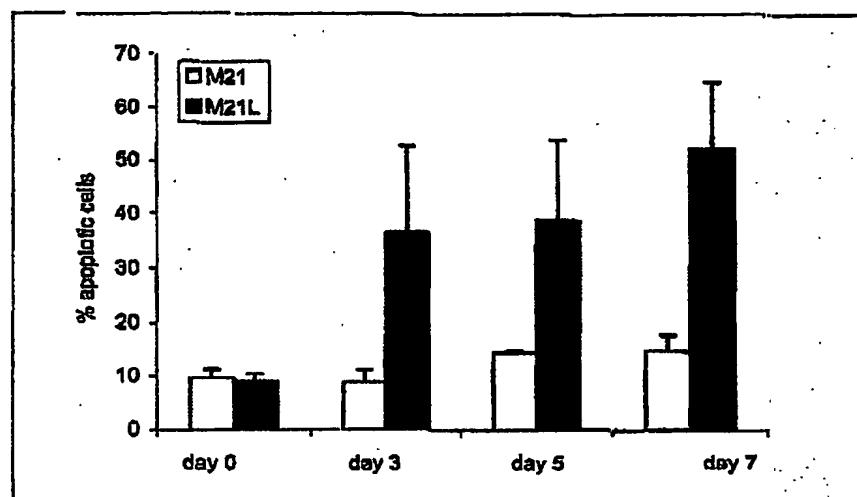
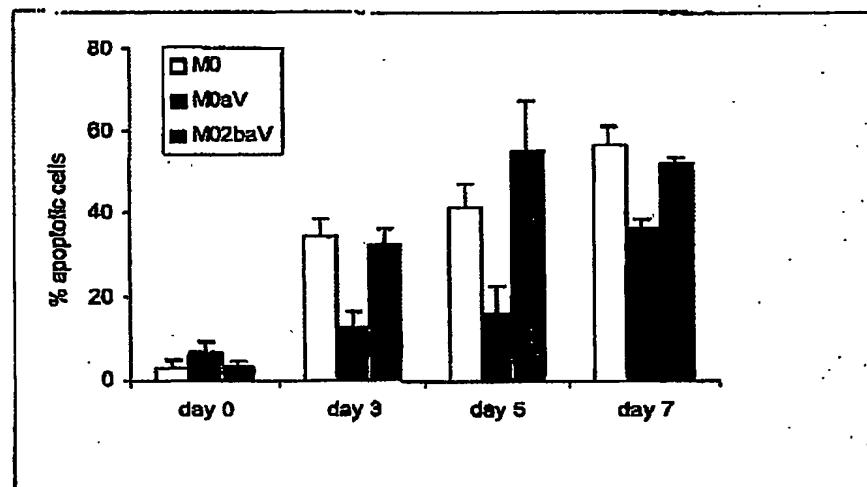


Figure 1B



46 8 4291070

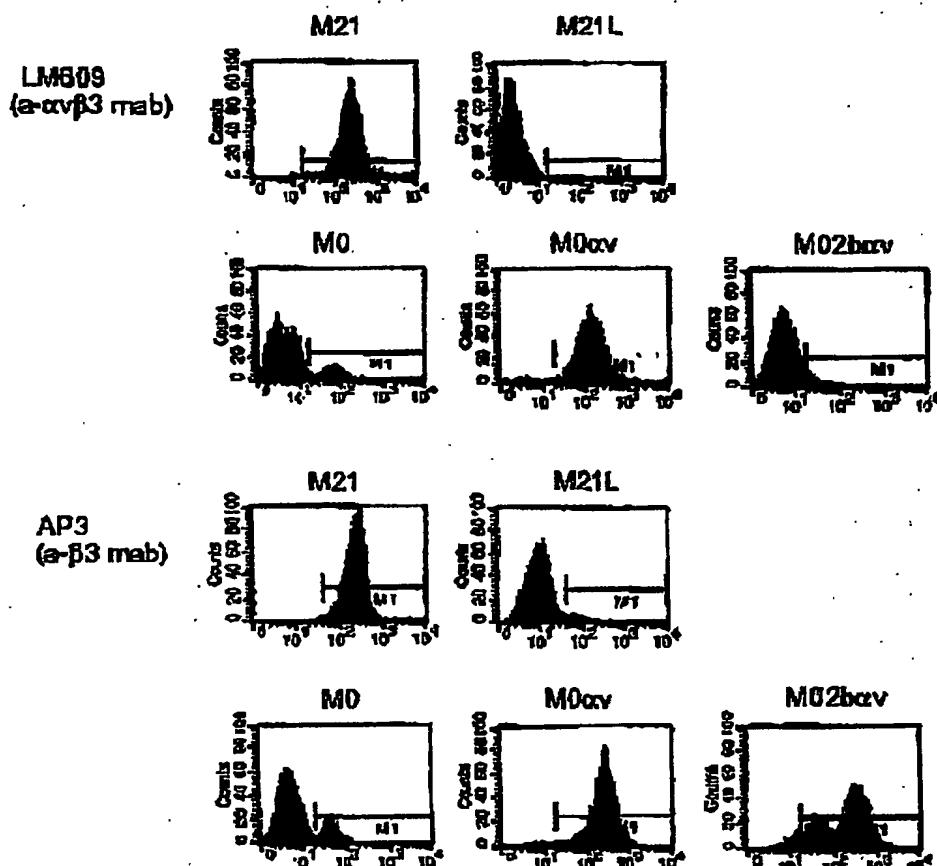
2/11

Ink. t. Patent- och reg.verket

2003-03-24

Huyudfaxen Kossan

Figure 1C



46 8 4291070

3/11

Figure 1D

Ink. t Patent- och reg.verket

2003-03-24

Huvudforsen Kassan

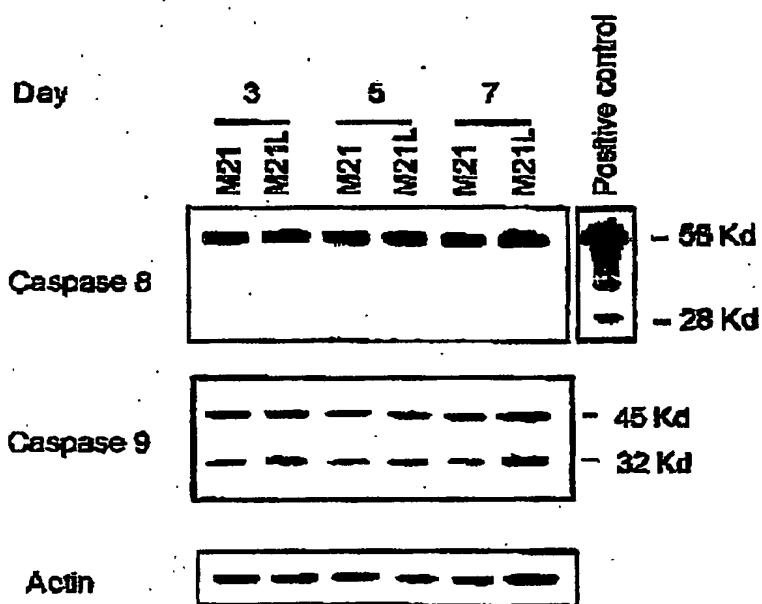
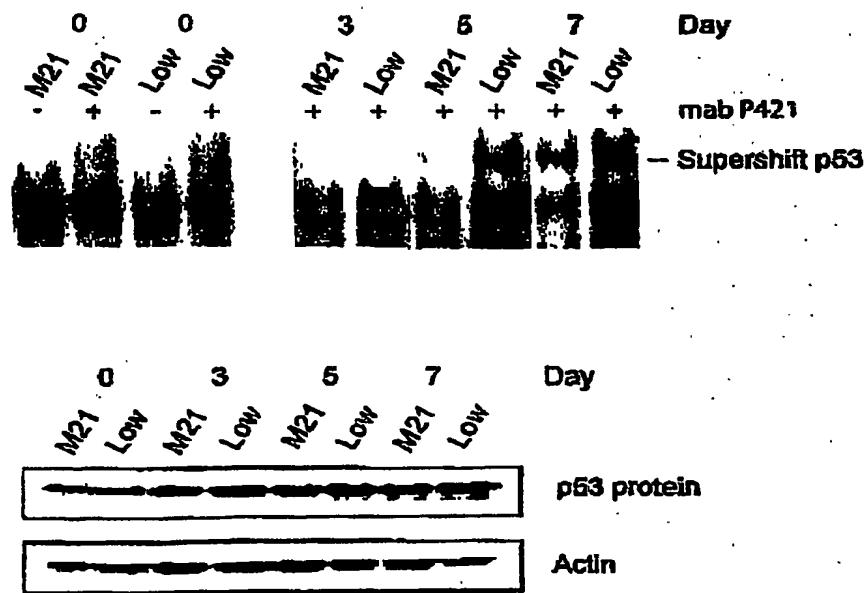


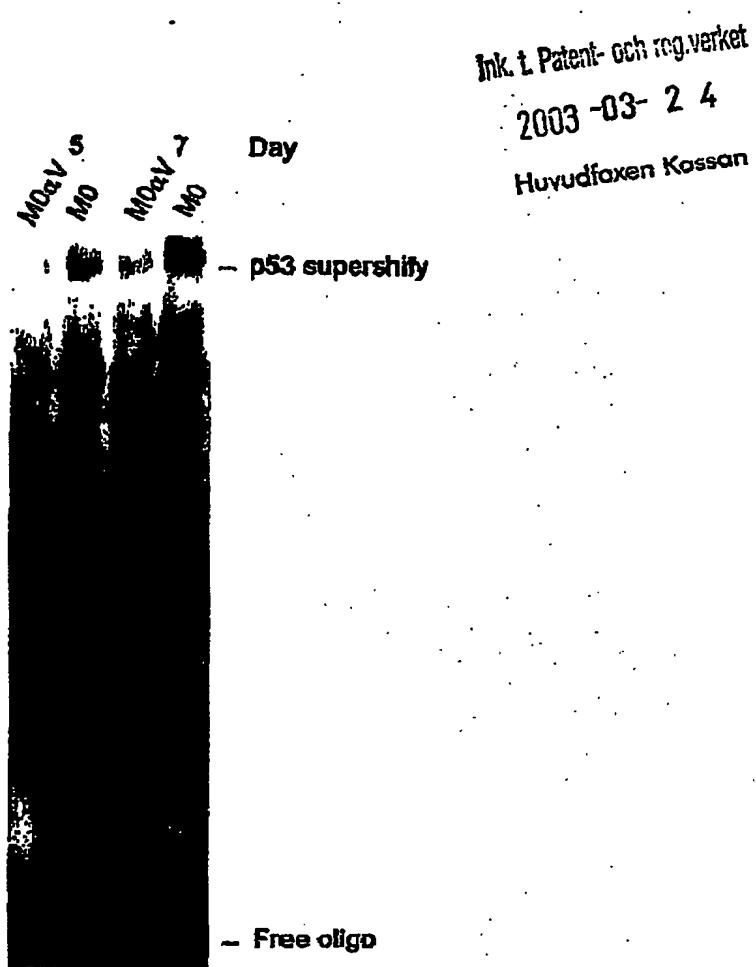
Figure 2A



46 8 4291070

4/11

Figure 2B



46 8 4291070

5/11

Figure 2C

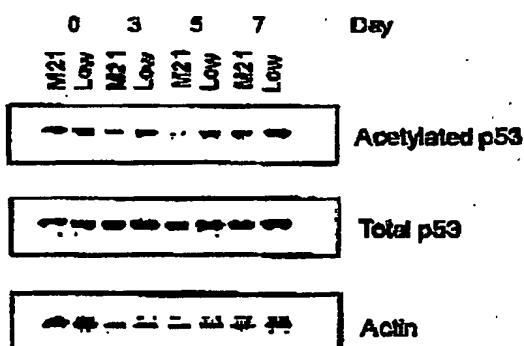
Figure 2

Ink t Patent- och reg.verket

2003-03-24

Huvudforsen Kassan

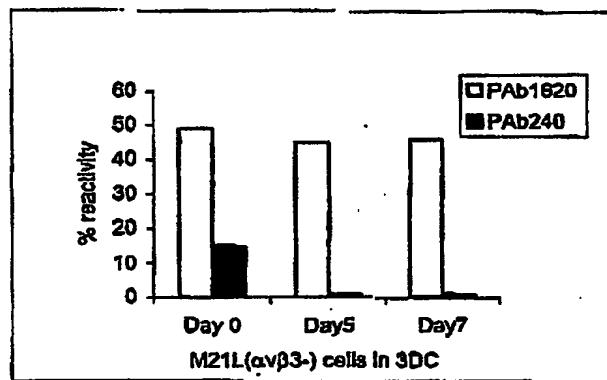
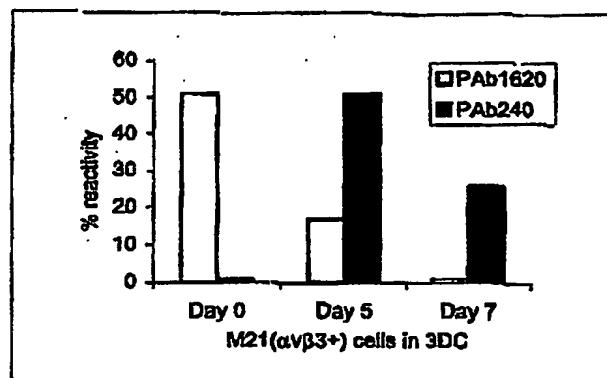
D



6/11

Figure 2D

Ink t Patent- och reg.verket
2003-03-24
Huvudfakten Kassan

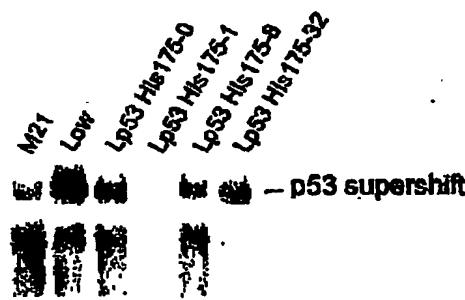
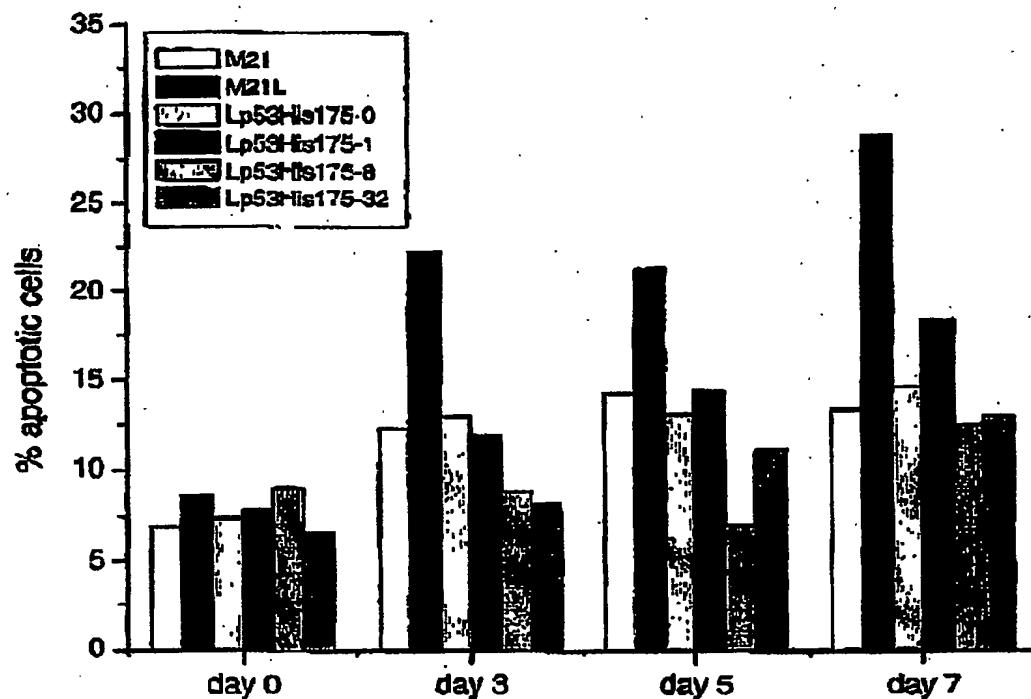


46 8 4291070

7/11

Figure 3A

Ink t Patent- och reg.verk
2003-03-24
Huvudfaxon Kassan



46 8 4291070

8/11

Ink t Patent- och PTJ-Verket
2003-03-24
Huvudfaxen Kassan

Figure 3B

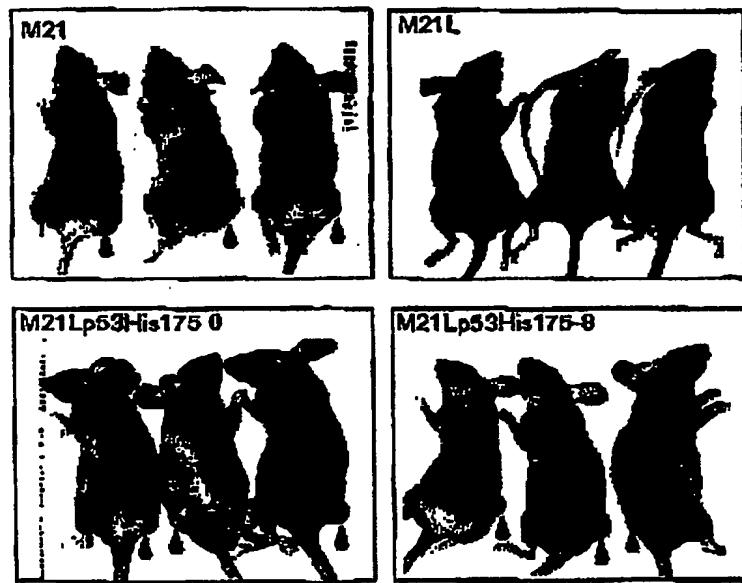
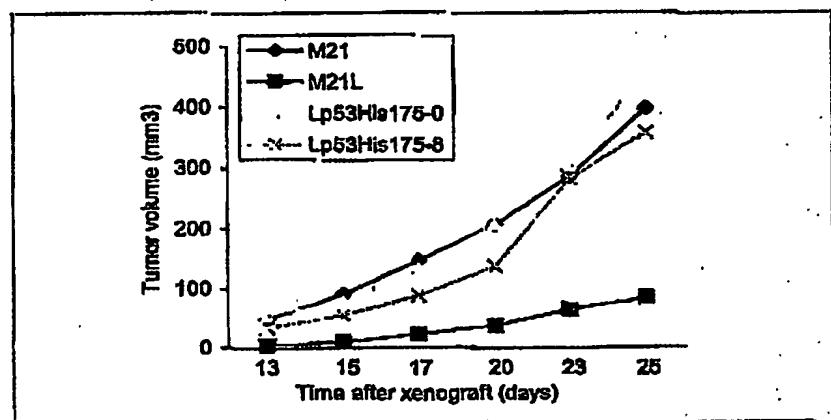


Figure 3C



46 8 4291070

9/11

Ink. t. Patent- och reg.verket

2003-03-24

Huvudfaxen Kassan

Figure 3D

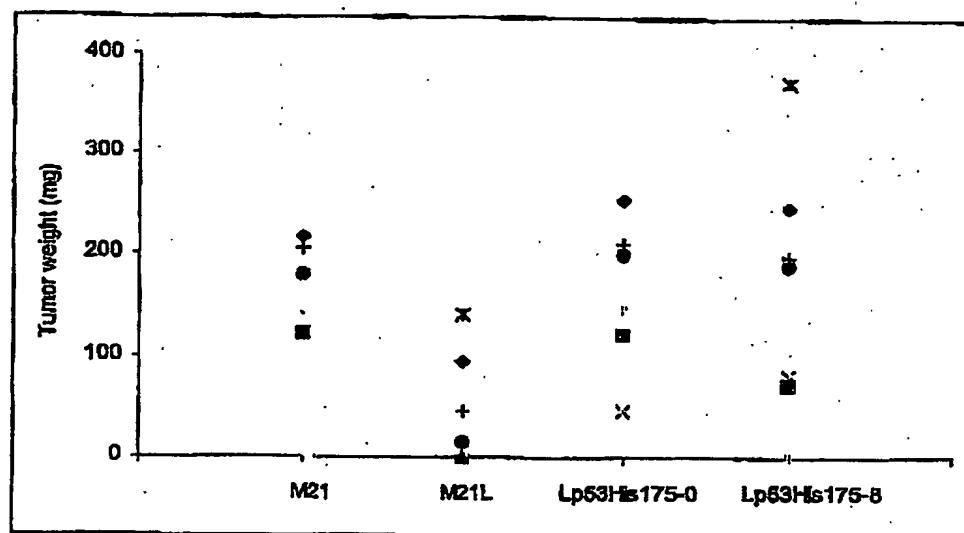
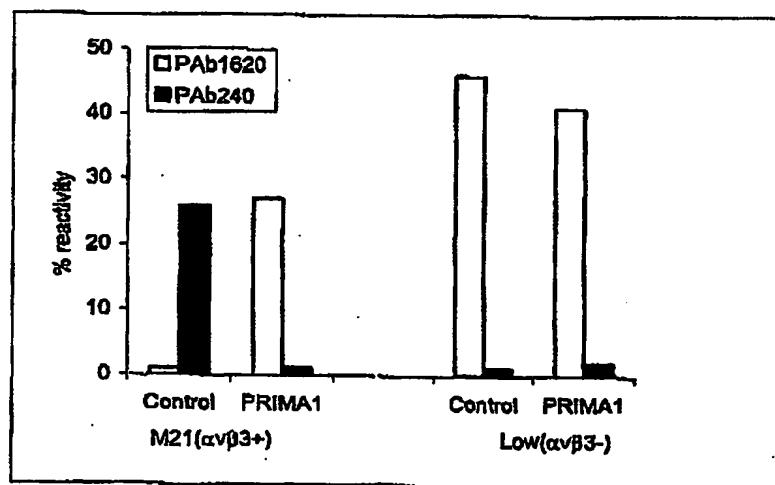


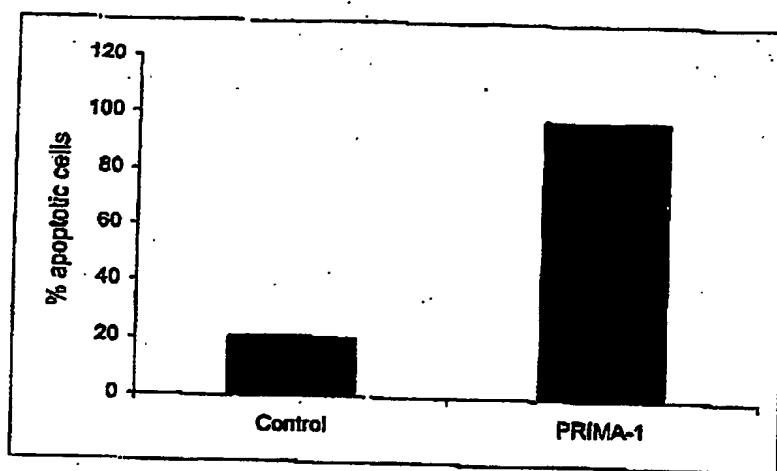
Figure 4A



46 8 4291070

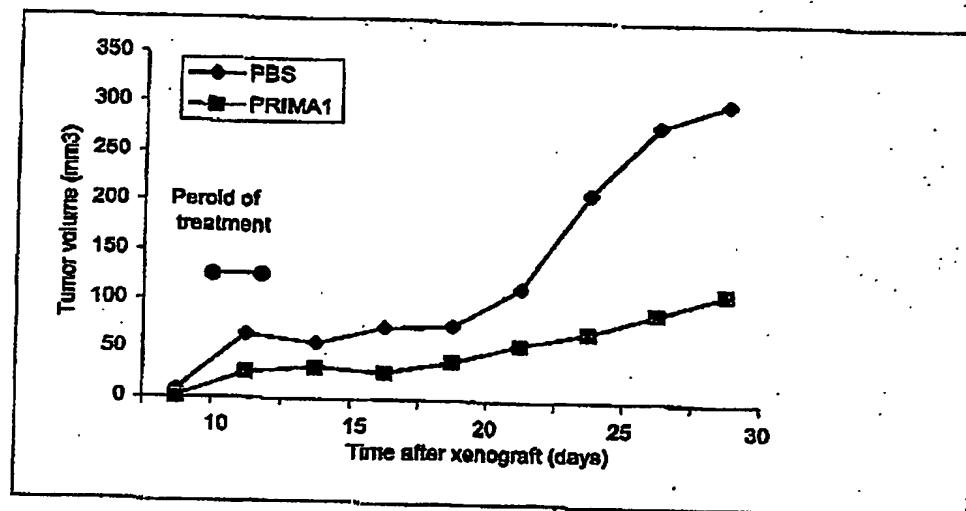
10/11

Figure 4B



Ink t Patent- och reg.verket
2003-03-24
Huvudfaxen Kassan

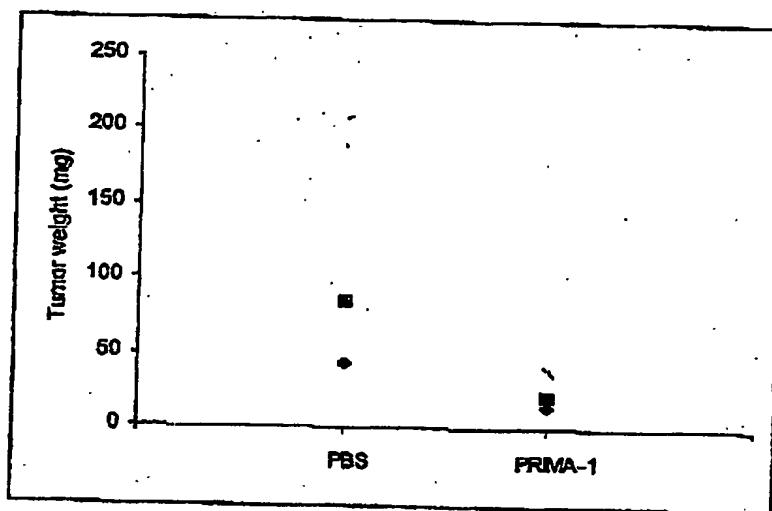
Figure 4C



46 8 4291070

11/11

Figure 4D



Ink. t. Patent- och reg. ver
2003-03-24
Huvudfaxen Kassan

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER: _____**

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.